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Specific Immunologic Tolerance to Dinitrofluorobenzene Following Topical Application of Dinitrothiocyanobenzene: Modulation by Suppressor T Cells

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In order to determine the mechanism(s) involved in the induction of immunologic tolerance for contact sensitivity via the topical application of a chemical that sensitizes if given with adjuvant, we utilized the hapten dinitrothiocyanobenzene (DNTB). Specific immunologic tolerance to dinitrofluorobenzene (DNFB) was induced in mice by the topical application of DNTB 7 days before sensitization to DNFB. The tolerance could be abrogated if cyclophosphamide (200 mg/kg) was given 3 days before attempted sensitization. Using passive transfer studies we found that DNTB induced hapten-specific Lyt 1^+2^- suppressor T cells. These suppressor cells prevented the induction of contact sensitivity but did not affect its expression. Lymphocyte proliferation studies, using haptenated epidermal cells as antigen, indicate that lymph node cells obtained 5 days after DNFB sensitization are far less responsive if the mice have received DNTB epicutaneously 7 days before the DNFB. Binding studies demonstrated that DNTB bound to epidermal cells at least as well as did DNFB. It is postulated that DNTB induction of suppressor cells is related to the physicochemical interaction between the hapten and antigen-presenting cells in skin.

Most studies of the mechanisms involved in immunologic unresponsiveness (tolerance) in contact sensitivity have used feeding or i.v. administration of various haptens in order to induce tolerance [1,2]. However, several studies have described the induction of tolerance in contact sensitivity following the epicutaneous (Epi) application of either the hapten itself or of a cross-reacting chemical. When the hapten is applied, either sub- or supraoptimal concentrations are used on normal skin [3–6] or it is applied directly onto skin that has been irradiated with small amounts of UV [7], or to abdominal skin of mice which have received large amounts of UV irradiation to their dorsal skin [8]. Using these methods, the animals are unable to become sensitized to the haptens even when the usual sensitizing dose is subsequently applied.

Other methods used for the induction of tolerance for contact sensitivity include the injection of the hapten s.c. without adjuvant [9], the injection of a cross-reacting nonsensitizing chemical, or the application of a chemical which sensitizes for contact sensitivity when it is emulsified with Freund's complete

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Abbreviations:
BSS: balanced salt solution
CY: cyclophosphamide
DNFB: dinitrofluorobenzene
DNP: dinitrophenyl
DNTB: dinitrothiocyanobenzene
EC: epidermal cell(s)
Epi: epicutaneous (application)
FCA: Freund's complete adjuvant
LNC: lymph node cells
TNCB: trinitrochlorobenzene (picryl chloride)

adjuvant (FCA) but cannot sensitize when applied Epi. In this regard, Friedlaender and Baer [10] and Sommer et al [11] reported that when dinitrothiocyanobenzene (DNTB) is applied to guinea pig skin, it induces a state of tolerance to DNTB or to the cross-reacting dinitrofluorobenzene (DNFB), a potent contact sensitizer. The present study was performed in order to determine the mechanisms involved in this unresponsiveness. We found that, in mice, DNTB application induces hapten-specific Lyt 1^+2^- suppressor T cells which act at the induction phase of contact sensitivity.

MATERIALS AND METHODS

Mice

BALB/c AnN female mice were obtained from the Goodwin Institute for Cancer Research Inc. (Plantation, Florida) through the Animal Genetics and Production Branch, DCT, NCI. Groups of 5–6 mice 8–12 weeks of age were used.

Antigens

2,4-Dinitrofluorobenzene (DNFB, Eastman Kodak Co., Rochester, New York), 2,4-dinitrothiocyanobenzene (DNTB, ICN Pharmaceuticals, Inc., Plainview, New York), and 2,4,6-trinitrochlorobenzene (TNCB, Tokyo Kasei, Tokyo, Japan) were used.

Sensitization and Elicitation for Contact Sensitivity

DNFB: Twenty microliters of 0.5% DNFB in 4:1 acetone:olive oil was painted on the shaved abdomen on consecutive days (days 0 and 1). Five days later (day 5), 20 μl of 0.2% DNFB in the same vehicle was applied to the dorsum of the ear and the increment in ear thickness, quantitated with an engineering micrometer, was evaluated 24 h later [6]. Animals that were challenged but not sensitized were used as negative controls.

DNTB in Freund's complete adjuvant (DNTB/FCA): Fifteen milligrams of DNTB was dissolved in 0.5 ml acetone and added to 7.5 ml of FCA (Difco Laboratories, Detroit, Michigan) and 7 ml of saline. They were emulsified as described by Sommer et al [11]. Two-tenths of a milligram of this emulsion (containing 0.2 mg of DNTB) was injected into the inguinal areas s.c. Seven days later 20 μl of 0.2% DNFB was applied to the ear and ear swelling was assessed 24 h later.

TNCB: One hundred microliters of 7% TNCB in 4:1 acetone:olive oil was painted on the shaved abdomen and 6 days later 20 μl of 1% TNCB in olive oil was applied to both sides of the ear. The increment of ear thickness was assessed 24 h later.

Induction of tolerance: One hundred microliters of 2% DNTB in acetone was applied to the hair-plucked back of mice 7 days before attempted sensitization [11]. These mice were then sensitized according to the sensitization schedules above and the degree of contact sensitivity assessed.

The effect of cyclophosphamide (CY) on tolerance induced by DNTB application: CY (Cytoxan, Mead Johnson Laboratories, Evansville, Indiana) was injected i.p. in a dose of 200 mg/kg either 3 days before application of the tolerogen, DNTB, or 3 days before application of the sensitizer, DNFB.

Transfer of tolerance: Lymph nodes were taken 7 days after application of DNTB or 6 days after application of TNCB and cell suspensions prepared. Nonsensitized syngeneic mice were injected i.v. with these cells; 1 h later sensitization with DNFB was attempted and 5 days later the mice were challenged. In other experiments the lymph node cells (LNC) were injected i.v. 1 h before attempted challenge of previously sensitized recipients.

Serum transfer: One week after application of DNTB mice were exsanguinated. Serum (1 ml) was injected i.v. into naive recipients which were then sensitized with DNFB.

Identification of suppressor cells: LNC were treated with either anti-theta (thy 1.2) alloantisera (Litton Bionetics), or with anti-Lyt 1.2 or anti-Lyt 2.2 monoclonal antibodies (New England Nuclear) and subsequently treated with complement (from 3-week-old rabbits). LNC treated with complement alone were used as controls.

Assessment of tolerance and reversal of tolerance

$$\% \text{ Suppression (unresponsiveness)} = \left(1 - \frac{\text{experimental} - \text{negative control}}{\text{positive control} - \text{negative}}\right) \times 100$$

$$\% \text{ Reversal of tolerance} = \frac{(\text{Experimental} - \text{tolerant control})}{(\text{Positive control} - \text{tolerant control})} \times 100$$

Statistical analysis: Each experiment was performed at least 3 times. Results are expressed as increment in ear thickness ± SEM. Student's *t*-test was used to assess differences in reactivity. A p value of less than 0.01 was considered significant. Representative results are presented.

Antigen-Specific Proliferation Assay

Cell preparation: LNC were obtained from nonsensitized mice, from mice sensitized to DNFB 5 and 4 days earlier, from mice which had DNTB applied 7 days earlier, or, from mice which received DNTB 12 days earlier and had DNFB applied 5 and 4 days earlier. Epidermal cell (EC) suspensions were prepared (from ear skin) by trypsinization [12].

EC conjugation with DNFB (DNFB-EC): EC were washed 3 times with Hanks' balanced salt solution (BSS) and incubated for 10 min with 0.05 mM DNFB at 37°C. The cells were then washed 3 times with Hanks' BSS containing 10% fetal calf serum.

Proliferation assay: Cells were cultured in RPMI 1640 containing 10% horse serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml fungizone, 5×10^{-5} M 2-mercaptoethanol, 10 mM HEPES buffer, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acid for 4 days in a humidified atmosphere of 92% air and 8% CO_2 at 37°C using 96-well flat-bottom microtest plates. As responder cells 2×10^5 LNC/well were used, and irradiated (2000 R) DNFB-EC, EC, DNFB-conjugated spleen cells or spleen cells alone were used in various concentrations (from 0.5×10^5/well to 2.0×10^5/well) as stimulator cells. One microcurie of [^{3}H]dThd was added to each well in the final 20 h of culture. Cells were harvested with a MASH harvester and radioactivity was assessed by liquid scintillation counting.

Enumeration of Dinitrophenyl (DNP) Groups per Epidermal Cell

The number of DNP groups per epidermal cell was determined using a modification of the method used by Hale for TNP [13]. Epidermal cell suspensions were prepared [12] and the cells were washed and then incubated with either DNFB (0.5 mM) or DNTB (.02 mM) for 30 min at room temperature. The haptenated cells were then extensively washed with Hanks' BSS containing fetal calf serum and 10^8 viable cells were incubated in 1 ml phosphate-buffered saline with 0.5% Nonidet P-40 for 10 min at room temperature. Nuclei and debris were separated by centrifugation (100,000 *g*) for 10 min. The supernatants were removed and clarified by the addition of sodium deoxycholate (final concentration 1%) and absorbancy at 350 nm was determined. Cells that had not been dinitrophenylated were used as a blank to correct for absorbancy.

RESULTS

Induction of Contact Sensitivity with DNFB or DNTB

DNFB (0.5%) applied on consecutive days regularly induced strong contact sensitivity. On the other hand, DNTB induced contact sensitivity only when emulsified with FCA and given s.c. When DNFB or DNTB were applied or when DNTB was given with FCA, mice challenged with 20 μl of 1% DNTB in acetone did not show any significant increment in ear thickness. Contact sensitivity could be elicited only with DNFB. Thus, cross-reactivity between DNFB and DNTB in contact sensitivity was recognized only when mice were sensitized with DNTB in FCA and challenged with DNFB (Fig 1).

Tolerance Induced by Epicutaneously Applied DNTB

To induce immunologic tolerance, 2% DNTB was applied to the hair-plucked back 7 days before the usual sensitizing dose of DNFB (0.5% on 2 consecutive days) was used. DNFB-sensitized mice which had prior DNTB treatment were significantly less sensitive (65–80% unresponsive) than those which had not had prior DNTB (Fig 2). Two applications of 2% DNTB, 14 and 7 days before attempted sensitization did not alter the level of unresponsiveness. As well, mice receiving DNTB in FCA were less sensitive to DNFB (52% unresponsive) if they received DNTB Epi before sensitization.

Hapten Specificity of DNTB-Induced Tolerance

To determine whether the tolerance induced by DNTB application was hapten-specific we sensitized mice to the noncrossreacting (to DNFB) hapten TNCB. DNFB-sensitized mice which had prior DNTB treatment were significantly less sensitive (71% unresponsive) than those which had not received prior DNTB treatment. This is in sharp contrast to the lack of effect of DNTB application on the subsequent ability to sensitize to TNCB (Fig 3).

Time Course for Induction of Tolerance by Epicutaneous Application of DNTB

To determine the optimal time for the induction of tolerance with DNTB, 2% DNTB was applied Epi 14, 10, 7, and 4 days before DNFB sensitization. DNTB applied 14 days before DNFB sensitization did not affect contact sensitivity. Although DNTB applied 10 and 4 days before DNFB sensitization induced significant tolerance, DNTB applied 7 days before DNFB sensitization induced optimal tolerance (68% unresponsiveness) (Fig 4).

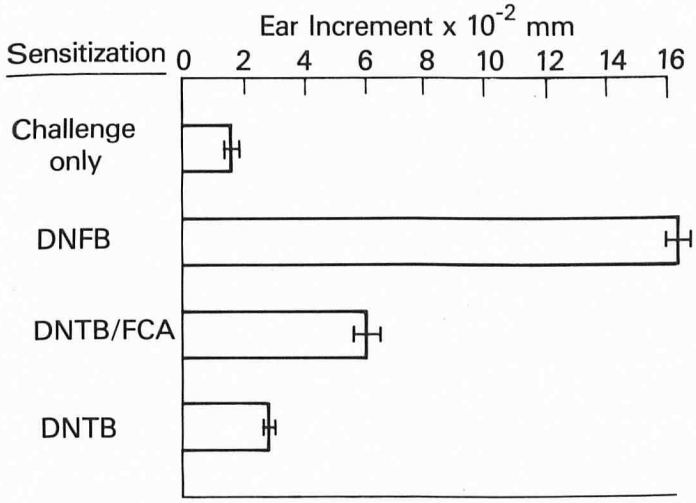

FIG 1. Increases in ear thickness 24 h after the application of 0.2% DNFB to mice to which (1) 0.5% DNFB had been applied 4 and 5 days earlier, (2) DNTB emulsified with FCA was injected into the inguinal areas 7 days earlier or (3) 2% DNTB was applied 7 days earlier.

Effect of CY on Tolerance Induced by DNTB

Two protocols were used in order to determine whether treatment with CY affected the tolerance induced by DNTB. In the first we gave CY i.p. 3 days before application of DNTB. Using this protocol we found that CY prevented the tolerance induced by DNTB by only 23 ± 8%. In the second protocol we gave CY i.p. 3 days before sensitization with DNFB (4 days after DNTB application) and found that CY significantly prevented the tolerance induced by DNTB (67 ± 15% reversal of tolerance). A representative experiment is depicted in Fig 5. These findings suggested that the unresponsiveness induced by DNTB might be due to its induction of suppressor cells.

Transfer of tolerance with LNC

To determine whether the tolerance induced by DNTB was transferrable with cells, LNC were obtained from mice which had received Epi DNTB 7 days earlier. LNC from mice sensitized with Epi TNCB (6 days earlier) were used as controls. LNC were injected i.v. into nonsensitized mice which then received the usual sensitizing dose of DNFB. The recipients were challenged on the ear 5 days later with DNFB. In other experiments, groups of recipient mice were sensitized with DNFB 5 and 4 days before receiving the LNC i.v. They were challenged on the ear 1 h later.

LNC from mice which had received Epi DNTB, injected into nonsensitized mice, prevented the recipients from becoming

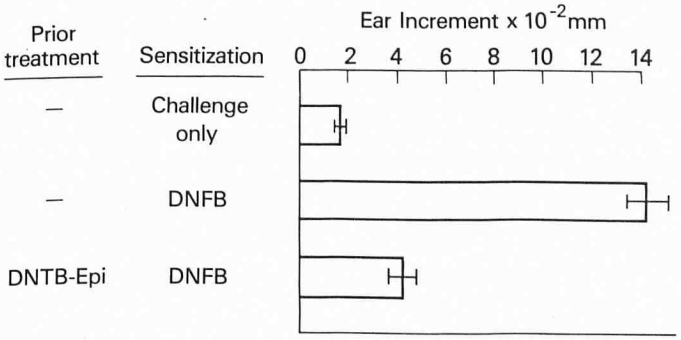

FIG 2. Increases in ear thickness 24 h after application of 0.2% DNFB mice which were (1) sensitized with DNFB 4 and 5 days earlier or (2) sensitized with DNFB 4 and 5 days earlier and had DNTB applied 12 days earlier (DNTB-Epi).

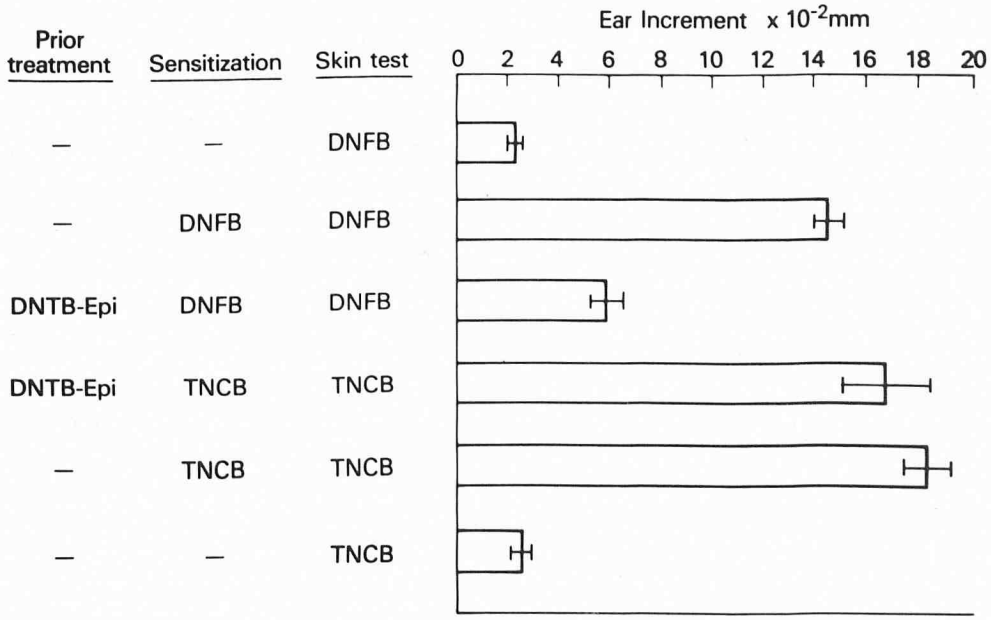

FIG 3. Mice received the usual sensitizing dose of DNFB or TNCB. Certain groups received DNTB epicutaneously (DNTB-Epi) 7 days earlier. Ear challenge was performed with the appropriate hapten 5 days (in the case of DNFB) or 6 days (TNCB) later.

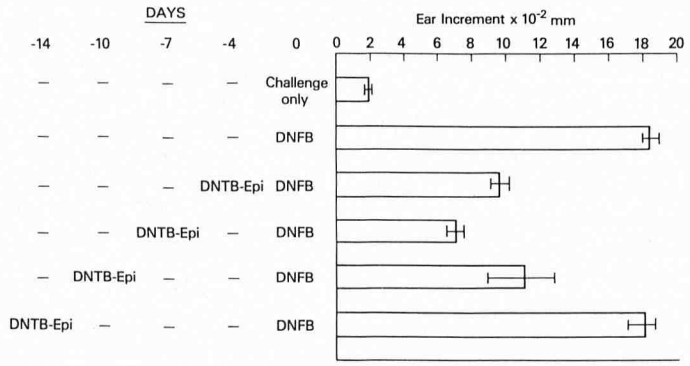

FIG 4. DNTB (2%) was applied epicutaneously at varying intervals before attempted sensitization with DNFB. Increases in ear thickness were assessed 24 h after challenge.

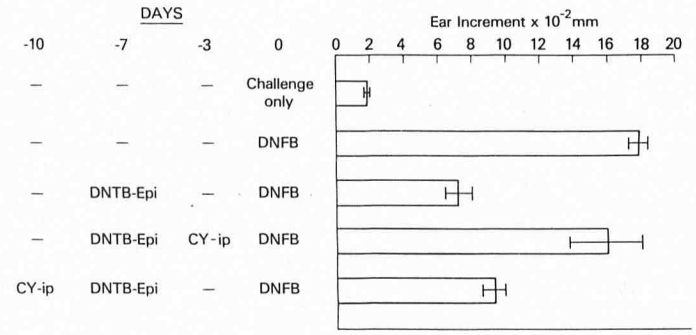

FIG 5. CY (200 mg/kg) was injected i.p. at varying intervals before or after application of DNTB. Sensitization was attempted and increases in ear thickness were assessed 24 h after challenge.

sensitive (Fig 6). This passive transfer of tolerance was dose (of LNC)-dependent and 4×10^7 viable LNC was optimal; this number was therefore used for all subsequent LNC transfers. LNC (4×10^7) obtained from mice sensitized with TNCB did not affect sensitization with DNFB, nor did heat-killed nonviable LNC from mice which had received Epi DNTB (Fig 6). LNC injected into previously sensitized mice did not affect skin test reactivity (data not shown). Serum from mice which had received DNTB did not affect sensitization to DNFB (data not shown).

The Effect of LNC Treated with anti-theta, anti-Lyt 1.2 or anti-Lyt 2.2 and Complement on Tolerance Induced by Epi DNTB

In order to determine whether the cells which transferred tolerance were T cells, the LNC were treated with anti-theta alloantisera and complement and 4×10^7 viable LNC were injected i.v. into nonsensitized mice. Cells treated with complement alone were used as controls. Using anti-theta (and complement)-treated LNC, DNFB sensitization was not affected, which is in sharp contrast to the effect of complement (alone)-treated cells which significantly suppressed DNFB sensitization (Fig 7). Anti-Lyt 2.2 and complement treatment of the transferred LNC did not affect their ability to suppress DNFB sensitization; however anti-Lyt 1.2 and complement treatment of transferred LNC abrogated the ability of the cells to suppress DNFB sensitization (Fig 8). Using either of these anti-Lyt monoclonal antibodies with complement in lymphocyte proliferation assays (antigen and mitogen), proliferative responses were markedly decreased; anti-Lyt 2.2 and complement treatment of responder cells also abrogated the ability of haptenated cells to generate a cytotoxic T-cell response (data not shown).

Lymphocyte Proliferation Assay

LNC from mice sensitized to DNFB Epi proliferated in a dose-dependent manner to DNFB-EC. Normal LNC did not respond to DNFB-EC. LNC from mice which received DNTB Epi before attempted DNFB sensitization showed a markedly diminished response to DNFB-EC. LNC from mice which had received Epi DNTB showed a significant proliferation response

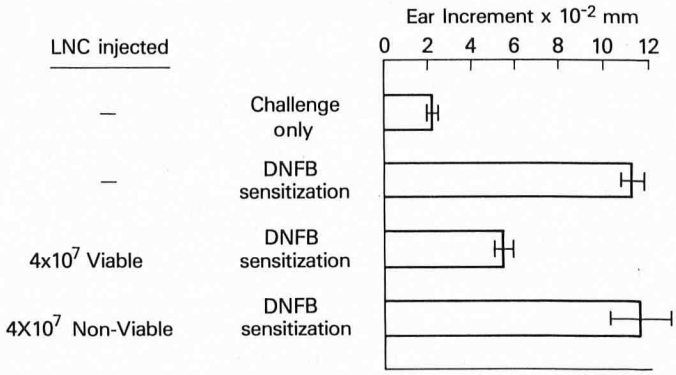

FIG 6. LNC from mice which had received epicutaneous DNTB 7 days earlier were injected into naive recipients in which DNFB sensitization was attempted 1 h after injection. Five days later, ears were challenged and 24 h thereafter ear thickness assessed.

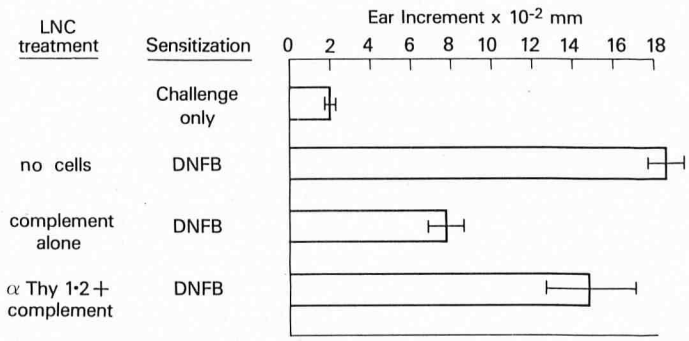

FIG 7. LNC were treated with complement alone or with anti-Thy 1.2 antiserum and complement and were then injected into naive recipients in which DNFB sensitization was attempted. Five days later ears were challenged and 24 h thereafter ear thickness assessed.

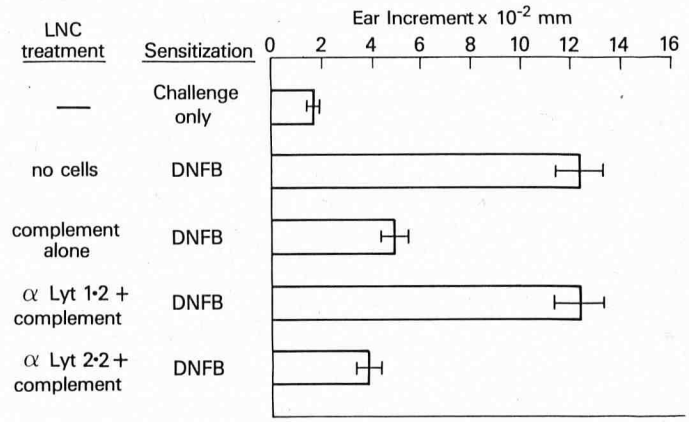

FIG 8. LNC were treated with complement alone or with anti-Lyt 1.2 or anti-Lyt 2.2 monoclonal antibodies and complement and were then injected into naive recipients in which DNFB sensitization was attempted. Five days later, ears were challenged and 24 h thereafter ear thickness assessed.

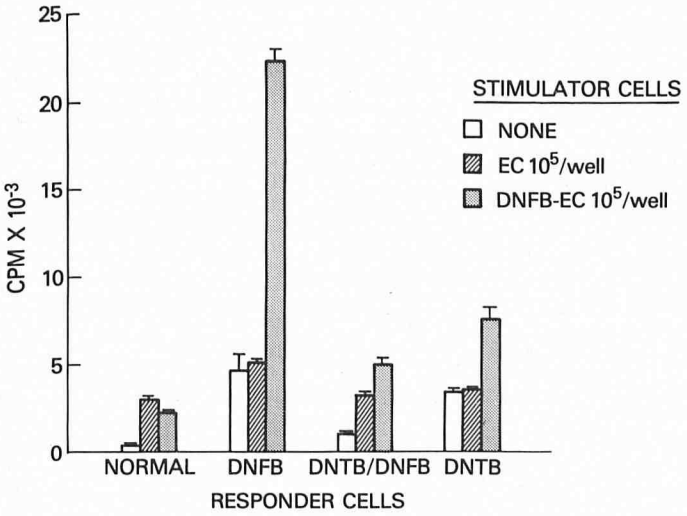

FIG 9. LNC from (1) normal mice, (2) mice sensitized to epicutaneous DNFB, (3) mice to which DNTB was applied 12 days earlier and DNFB applied 5 days earlier, or (4) mice which received DNTB alone were used as responder cells and epidermal (EC) or DNFB-conjugated EC (DNFB-EC) were used as stimulators in a 4-day culture. [^{3}H]dThd was added in the final 20 h of culture. Cells were harvested with a MASH harvester, and radioactivity was assessed by liquid scintillation counting.

to DNFB-EC when compared to stimulation with EC alone but showed a markedly diminished response compared to LNC from mice sensitized to DNFB but not pretreated with DNTB (Fig 9). Maximal responses were seen on days 4 and 5 of culture.

Enumeration of DNP Groups per Epidermal Cell

In order to determine whether epidermal cells were conjugated as well with Epi DNTB and DNFB, we made single EC suspensions and conjugated them in vitro. The number of DNP groups per EC was 2×10^7 when DNTB was used whereas it was 10^7 when DNFB was used.

DISCUSSION

Specific immunologic tolerance in contact sensitivity can be induced in a variety of ways. Generally, if a chemical is a sensitizer when given by one route, i.e., Epi or s.c., and is given by a different route i.e., i.v., i.p., or by feeding, it no longer sensitizes but induces tolerance (reviewed in [1,14]). As well, a chemical that is related to a sensitizer but modified in one of several ways can often induce tolerance to the sensitizing chemical. Thus tolerance to poison ivy can be induced in animals given any one of a series of derivatives substituted in the 6 position of the pentadecylcatechol structures [15]. Also dinitrobenzenesulfonate [16] and dichloronitrobenzene [17] induce tolerance to DNFB or dinitrochlorobenzene. Another substance which regularly induces tolerance to DNFB is DNTB. Baer and his coworkers [10,18] suggested that since DNTB was less reactive with proteins such as bovine serum albumin, it was lost from the sensitizing depot and entered the blood and induced tolerance in a manner similar to that described by Macher and Chase [19,20]. Sommer et al [11] subsequently demonstrated that in guinea pigs, DNTB induced a state of partial unresponsiveness to DNFB by generating CY-sensitive suppressor cells and that these cells acted by competition with effector cells at the periphery. The unresponsiveness was not associated with a decrease in T-cell proliferation in the draining lymph nodes.

In this study we showed that DNTB application induces hapten-specific suppressor T cells whose generation is prevented by treatment with CY prior to attempted sensitization. When passively transferred into naive recipients, these cells suppress the recipients' ability to become sensitized to DNFB. The suppression is at the induction and not at the expression phase of contact sensitivity. The lymphocyte proliferation studies indicate that LNC obtained 5 days after DNFB sensitization are far less responsive if the animals received DNTB Epi 7 days before the DNFB. Thus DNTB application induces T cells which block the capacity of other T cells to become responsive to the cross-reacting DNFB. Interestingly, when DNTB is given with FCA, it is a sensitizer.

Epicutaneously applied hapten has been previously shown to induce specific immunologic tolerance. Asherson et al [5] and Sy et al [6] have demonstrated that sub- or supraoptimal doses of epicutaneously applied hapten can prevent sensitization when the same hapten is subsequently applied at optimal concentration. Both groups demonstrated that suppressor T cells were induced, however, in contrast to those generated in this study, CY treatment of the mice 3 days before the initial hapten application abrogated the unresponsiveness, whereas CY treatment before DNTB application prevented the tolerance induced by DNTB by only 23%. When CY was given 3 days before attempted sensitization with DNFB the tolerance induced by DNTB was significantly abrogated (67%). The precise mechanism(s) through which CY works in this model is unknown.

It is unlikely that DNTB was being lost from the sensitizing depot and entering the blood by virtue of its chemical and physical properties as it is highly insoluble in water. Binding studies demonstrated that DNTB was able to bind to EC as well as DNFB. Thus cells incubated with DNTB were even more densely haptenated than were those incubated with DNFB. It may be that tolerance induction with DNTB is somehow related to the chemical groups on the surface of epidermal cells (and in particular, Langerhans cells) with which the DNTB reacts. It is known, for example, that DNTB reacts with sulfhydryl-containing amino acids at neutral pH in vivo [21], whereas DNFB reacts mainly with ε-amino groups [22, 23]. However, related compounds such as DNCB also react mainly with sulfhydryl-containing amino acids and are very potent sensitizers when applied to the skin.

A possible explanation as to why suppressor cells are generated following DNTB application may be related to the interaction between the hapten and the antigen-presenting cells (Langerhans cells) in skin. DNTB may bind to the Langerhans cells or alter the cell surface in such a way as to facilitate its induction of suppressor cells either in the periphery or even in the draining lymph nodes. It is known for example that one can alter Langerhans cells (with UV radiation) so that when they are haptenated (with a normally sensitizing chemical) they will generate suppressor T cells [24].

Our studies are in keeping with others [25,26] in which suppressor T cells may act at the induction rather than expression phase of the contact sensitivity. It may be that these suppressor cells inactivate precursor T helper cells or induce other feedback suppressor cells [27]. Although in most experimental systems suppressor T cells are of the Lyt $1^-2^+3^+$ phenotype, there are numerous examples of suppressor T cells in delayed-type hypersensitivity bearing the Lyt 1^+2^- phenotype [25,28, 29]. Study of the interactions of skin, and especially of antigen-presenting cells in skin, with chemicals such as DNTB and with lymphoid cells may provide further insight into how these suppressor cells are generated.

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